PATENT
ATTORNEY DOCKET NO. 07004/002001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Jong Y. Lee
Serial No.: 08/850,293
Filed : 05/05/97
Title : PURIFIED HUMAN ERYTHROPOIETIN RECEPTOR PROTEIN FRAGMENT AND ANTIBODIES DERIVED THEREFROM

Art Unit: 1646
Examiner: D.Fitzgerald

Assistant Commissioner for Patents
Washington, DC 20231

DECLARATION UNDER 37 CFR 1.132

I, Jong Y. Lee, do hereby declare as follows:

1. That I am the inventor named in the above-identified application;

2. That I have read and understood Harris et al., J. Biol. Chem., 267(21):15205-15209, 1992 (Harris);

3. That I have read and understood the U.S. Patent and Trademark Office Final Action for U.S. patent application serial No. 08/850,293, mailed February 24, 1998;

4. That I constructed an EpoR cDNA recombinant pGEX3X vector and corresponding fusion protein duplicating, to the extent possible, the methodology published by Harris using the reagents, materials and methods described below.

Glutathione (GSH)-agarose, pGEX-3X expression vector and protease Factor Xa were obtained from Pharmacia Biotech

Date of Deposit April 23, 1998
I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.
Chantal R. Billadeau
Chantal R. Billadeau

(Mechanicsburg, PA). PCR reagents were obtained from Perkin-Elmer Cetus (Norwalk, CT) and Affigel® 15 was obtained from BioRad (Richmond, CA). Bacteriophage T4 DNA ligase, restriction enzymes and isopropylthio-β-D-galactoside (IPTG) were obtained from BRL Gibco (Gaithersburg, MD). Nitrocellulose was obtained from GermanSciences (Ann Arbor, MI), chemiluminescence (ECL) reagents and autoradiography film were obtained from DuPont-NEN Research (Boston, MA). Phenylmethylsulfonylfluoride (PMSF), diisopropylfluorophosphate (DFP), thrombin and Triton X-100 were obtained from Sigma (St. Louis, MO). Biotinylated rabbit anti-sheep antibodies and Avidin-horse radish peroxidase were obtained from Pierce Co. (Rockford, IL). Sheep antibodies were prepared in my laboratory using standard procedures. A full-length human Erythropoietin receptor (EpoR) cDNA (LAP37) was from Dr. Bernard G. Forget, Yale University. Oligonucleotides were synthesized by the microchemical facility of the Institute of Human Genetics, University of Minnesota, Minneapolis, Minnesota. All other chemicals were of reagent grade.

The extracellular domain of the full-length human EpoR cDNA (LAP37) was PCR amplified using the 5'-sense primer (5'-TGGATCCGGGCGCCCCCGCCTAAC-3': BamH1 linker + coding sequence) and the 3'-antisense primer (5'-TGAATTCGGGGTCCAGGTCGCT-3': EcoR1 linker + coding sequence).

Each PCR reaction contained approximately 0.1 μg of EpoR cDNA, 20 pM of each primer, 1.25 mM of a dNTP mixture (dGTP, dCTP, dTTP and dATP) and 0.5 μl of Taq polymerase with 10x buffer supplied in the Perkin Elmer-Cetus PCR kit. Amplification was

carried out on the Perkin-Elmer PCR System 2400 (Norwalk, CT), with 25 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1.5 minutes. The PCR product (approximately 600 bp) and pGEX-3X (approximately 5 kB) were verified on a 1% molecular biology certified agarose gel (Bio-Rad Laboratories, Hercules, CA) in 1x TA buffer, using Hind III and Hae III DNA standards.

The PCR product and the pGEX-3X vector were each digested with EcoR1 and BamH1 for 4 hours at 37°C and then gel-purified by the Geneclean method according to the manufacturer's instructions (FMC BioProducts, Rockland, ME). The PCR product was ligated into pGEX-3X plasmid vector using approximately 1 μg/μl each of PCR product and pGEX-3X expression vector and approximately 20 Weiss units of T4 DNA ligase at 16°C overnight. Both *E. coli* BL21 cells (supplied with pGEX-3X plasmid) and *E. coli* strain JM109 (purchased from American Type Culture Collection, Rockville, MD) were transformed (approximately 20 μl of ligation mixture/200 μl of bacteria). After a 48 hour incubation at 37°C, discrete colonies were cultured in order to harvest DNA for verification of insert sizes. Extracted DNAs were electrophoresed through a 0.8% agarose gel in TA buffer, stained with ethidium bromide (0.5 μg/ml) and verified by the Bio-Rad Gel Doc System. Colonies #46 and #64 (~5.5 kb) were identified as containing the successfully constructed EpoR recombinant vector.

I produced EpoR recombinant fusion protein from colonies #46 and #64. Colonies #46 and #64 were grown overnight

at 37°C with vigorous shaking in 400 ml of LB medium with 100 μg/μl of ampicillin. The following day, the cultures were diluted in 4 liters of fresh LB/amp media and incubated for another 90 minutes before adding 1 mM IPTG. After 4 hours of IPTG induction, the cells were pelleted at 3,000 x g at 4°C for 15 minutes and resuspended in 160 ml of lysis buffer containing 50 mM sodium phosphate, pH 7.4, 10 mM β-mercaptoethanol, 10 mM EDTA, pH 8.0, 1.15 mM PMSF and 1 mM DFP. Approximately 160 mg of lysozyme were then added to the suspension. A 60 cc syringe was used to homogenize the lysed cell suspension by passing the suspension through 18, 21, and 23 gauge needles three times and incubating on ice for 30 minutes. After three dry ice/methanol freeze-thaws at 37°C and mild sonication, 1% triton-X was added. Supernatant was collected by centrifugation at 15,000 x g at 4°C for 15 minutes.

EpoR fusion protein was purified by GSH affinity column chromatography as described in the present application. A GSH agarose column was prepared by washing swollen GSH-agarose beads three times with 10 bed volumes of phosphate-buffered saline (PBS). The column was equilibrated with 5 bed volumes of isotonic PBS. The supernatant was applied to the column and then washed twice with 10 bed volumes of PBS. EpoR fusion protein was eluted with 5 bed volumes of elution buffer containing 5 mM reduced GSH in 50 mM Tris-HCl, pH 8.0.

The purified proteins were electrophoresed through SDS-PAGE to verify their molecular weights. As shown in Figure 7 of the Declaration, fusion proteins from colonies #46 and #64 (lanes

2-3) are approximately 55 kD. EpoRex-th, from the present application, is also approximately 55 kD (lane 4);

5. That I have confirmed that the fusion proteins obtained by the methodology of Harris are not cleaved by factor Xa and that they retain the GST moieties. EpoR recombinant fusion proteins from colonies #46 and #64 were incubated at room temperature for 30 minutes or for 1 hour with 7.5 units or 15 units of factor Xa. As shown in Figure 8 of this Declaration, recombinant protein from colony #64 remains at 55 kD, irrespective of temperature and excess concentration of factor Xa. In Figure 8, "Xa frag." refers to factor Xa fragments (17-22 kD, 28-38 kD and about 42 kD) that appear when the disulfide bonds of factor Xa are reduced upon exposure to the SDS-buffer, and correspond with the description obtained from the manufacturer.

Factor Xa cleavage experiments were repeated with fusion proteins from colonies #46 and #64 and compared with thrombin cleavage experiments using EpoRex-th fusion protein from the present application. Cleavage reactions were carried out with 10 units of factor Xa or with 0.6 μg of thrombin at room temperature for one hour. As shown in Figure 9 of this Declaration, recombinant proteins produced from the methodology of Harris et al. remained as fusion proteins, despite the excess concentration of factor Xa (compare control lanes 2 and 4 with experimental lanes 3 and 5). Recombinant EpoRex-th was cleaved

into Epo-bp and GST with a minimal concentration of thrombin (lane 6).

I confirmed the results of the above-described cleavage experiments by Western blotting using anti-Epo-bp sheep antibodies. Anti-Epo-bp antibody recognizes the extracellular domain of the EpoR. Following a room temperature incubation of 10 units of factor Xa with EpoR produced by the methodology of Harris, the proteins were separated electrophoretically and transferred to nitrocellulose to see the cleavage. Anti-Epo-bp antibody (1:2000 dilution) was introduced and incubated at room temperature for 1 hour with gentle agitation. After the first antibody was washed off, a biotinylated rabbit antiimmunoglobulin-anti-sheep antibody (1:10,000 dilution) was incubated at room temperature for 1 hour, with rocking. This was followed by a 1 hour, room-temperature incubation with enzyme horse radish peroxidase-Avidin (1:10,000 dilution). After a brief soaking in ECL reagents, wet blots were exposed immediately to Kodak X-ray film.

As indicated in Figure 10a of this Declaration, EpoR protein produced by the methodology of Harris is not cleaved by factor Xa. Lanes 2 and 4 contain EpoR from colony #46 and colony #64, respectively, in the absence of factor Xa. Lanes 3 and 5 contain EpoR from colony #46 and colony #64, respectively, in the presence of factor Xa. EpoR protein was the same size with or without factor Xa cleavage.

The failure of factor Xa to cleave EpoR fusion protein produced by the methodology of Harris was reconfirmed by probing

the blot of Figure 10a with anti-GST antibodies. The blot of Figure 10a was stripped in stripping buffer containing 67 mM Tris-HCl, pH 6.7, 2% SDS, 0.67% β-mercaptoethanol and reprobed with anti-GST antibodies as described above. As shown in Figure 10b, EpoR protein produced by the methodology of Harris retains the GST moiety after incubation with factor Xa;

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and believe are believed to be true; and further that these statements were made with the knowledge that willful false statements and alike so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: April 23, 1998

Jong Y. Lee, Ph.D.

13823.M11